**Technological approaches to improve the quality of meat products**

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This research project dealt with the feasibility of using the Olive Leaf Extract (OLE) to limit the amount of nitrate and nitrite added in ripened sausages. The research activity has been developed, during the three years of the Ph. D course, in three steps: i) preliminary trial of ripened sausages production with different nitrate/nitrite and OLE ratios carried out at laboratory scale; ii) production of sausages with OLE added at industrial scale (*scale-up*); iii) evaluation of shelf-life of ripened sausages with OLE during MAP storage at 4°C.

Approcci tecnologici per migliorare la qualità dei prodotti carnei

Questo progetto di ricerca riguarda la possibilità di utilizzare l’estratto di foglie di olivo (OLE) per ridurre la presenza dei nitrati e nitriti nelle salsicce stagionate, responsabili della formazione dei composti N-nitroso. Il progetto è articolato in tre diverse fasi, tra cui: i) studio preliminare di produzione delle salsicce stagionate con diversi rapporti di nitrati/nitriti e OLE su scala di laboratorio; ii) produzione delle salsicce stagionate addizionate di OLE su scala industriale; iii) valutazione della shelf-life delle salsicce stagionate addizionate di OLE durante la conservazione in MAP a 4°C.

**Key words**: vegetable extract; nitrate and nitrite reduction; additives; ripened sausages; shelf-life evaluation.

# **1. Introduction**

Meat and meat products have an important role in human nutrition since they are source of noble proteins, provide all essential amino acids and various micronutrients. They are an iron source in the bio-available form and, therefore, better absorbable by human body, good levels of vitamins B6 and B12, vitamin D (Ferguson, 2010). Furthermore, they are part of the eno-gastronomic culture of several countries. However, medical and scientific studies have correlated the consumption of these products, especially of red and processed meat, with the development of various diseases in the cardiovascular system and to an increase in the risk of cancer (Turner and Lloyd, 2017). In fact, the high-fat intake, and/or carcinogens compounds generated through processing methods and the additive transformation may be responsible of these risks. Among meat additives, nitrate and nitrite are used to allow stability of the red colour, inhibition of the growth of undesirable bacteria, improvement of the oxidative stability and contribution to flavour formation (Flores and Toldrá, 2021). Nevertheless, the addition of these synthetic additives to processed meat can induce the development of N-nitroso compounds linked with genotoxicity and metabolic disturbances in the intestine mucosa, with the subsequent risk to potentially develop colorectal cancer (Jian *et* *al.,* 2019). For these reasons, over the years, the replacement of nitrate and nitrite by natural extracts in processed meat have been proposed as valuable alternative. Natural ingredients, essential oils, extracts from fruits and vegetables spices have been used as nitrite scavenging and inhibitors of N-nitroso compounds formation, such as nitrosamines (NAs) (Tian *et al.,* 2020). Furthermore, extracts from wastes and by-products of the agricultural and food industry sectors contain highly valuable bioactive substances such as polyphenols with antioxidant and antimicrobial activity. Thus, olive leaves have been proposed for food preservation thanks to the abundant bioactive molecules with antioxidant and antimicrobial activities, such as oleuropein (Difonzo *et al.,* 2017). Several studies showed that the addition of OLE improved the oxidative stability, exerted an antimicrobial effect, and extended the shelf-life of several foods. However, its application on meat-based products has been less studied. In the present oral communication, the main results of the feasibility of using OLE for the reduction of nitrate and nitrite level in the ripened pork sausages are reported. This objective has been developed according to three different experimental steps: A1) preliminary trial of ripened sausages production with OLE addition at laboratory scale; A2) production of ripened sausages with OLE added at industrial scale (*scale-up*); A3) shelf-life prediction of ripened sausages with OLE during storage in MAP condition at 4°C.

# **2. Material and Methods**

**2.1 Preliminary trial of ripened sausages production with OLE addition in laboratory scale**

For the preliminary trials in laboratory scale, an OLE extract, obtained with the procedure described in Difonzo *et al.* (2017) has been used. Pork meat was purchased from a local farm Salumi Martina Franca S.r.l. (Martina Franca, Italy). Sausages were manufactured using the lean meat and the adipose tissue (85/15, w/w) from extensively reared pigs. Potassium nitrate E252, sodium nitrite E250 (SolMar, Italy) and salt (40 g/kg of raw meat) were added. Six different formulations of sausages (F) with different nitrate, nitrite and OLE ratios were produced and compared to a control sample containing the maximum levels of nitrate and nitrite admitted by Reg. (UE) No 1129/2011. Control (OLE: 0 mg/kg; NO2-NO3: 150-150 mg/kg); F1 (OLE: 200 mg/kg; NO2-NO3: 75-75 mg/kg); F2 (OLE: 400 mg/kg; NO2-NO3: 75-75 mg/kg); F3 (OLE: 800 mg/kg; NO2-NO3: 75-75 mg/kg); F4 (OLE: 200 mg/kg; NO2-NO3: 0 mg/kg); F5 (OLE: 400 mg/kg; NO2-NO3: 0 mg/kg), F6 (OLE: 800 mg/kg; NO2-NO3: 0 mg/kg). After ripening the nitrate and nitrite residual content, sulphite *Clostridia* and spores, *Coliforms, Escherichia coli* and *Staphylococcus* coagulase positive, were carried out following the methods described in Difonzo *et al.* (2022).

**2.2 Production of ripened sausages with OLE added at industrial scale (*scale-up*)**

Based on the results obtained from the first experimental trials, a *scale-up* approach was also performed. In this case, a commercial OLE (Olive Leaf Extract, Hepatica, Germany) commonly marketed as dietary supplement, at 40% of oleuropein content, was used for the production Ripened sausages were manufactured at the local farm Salumi Martina Franca S.r.l. sited in Martina Franca (Taranto, Italy) following common industrial processing applied by the company. The raw meats were minced by an industrial meat grinder equipped with a pre-mixer. In this case, five different formulations (F) with different nitrate, nitrite and OLE ratios were considered (Table 1).

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Table 1 *Formulations of OLE – nitrate and nitrite used for samples*  *preparation during the scale-up.* | | | | | |
| Sample | **F1** | **F2** | **F3** | **F4** | **F5** |
| Olive Leaf Extract (mg/kg) | 1000 | 1000 | 0 | 0 | 500 |
| Nitrate and nitrite (mg/kg) | 0 | 75 NO2  75 NO3 | 0 | 75 NO2  75 NO3 | 35 NO2  35 NO3 |

During mechanically kneading, salt (20 g/kg) and pepper (1 g/kg) were also added. The mixture was mechanically stuffed into natural pork casings and submitted to stewing (23°C, RH 95%, 24 h) to activate the fermentation process, drying (17-20 °C, RH 60-75%, 96 h) and ripening (15-18 °C, RH 80%). At the end of ripening period, physicochemical analysis, colour parameters, lipid oxidation and nitrosamines content were evaluated.

**2.2.1 Weight loss, water activity (aw), pH, moisture content**

Weight loss (%) was calculated as percentage of differences in weight of the whole sausages between day 0 and the end of ripening time. The pH was measured by inserting the glass pH electrode in the meat portion (HANNA instruments, Woonsocket, USA) and the aw was determined using a hygrometer (Aqua Lab 100–240 V AC, Pullman, USA). The determination of moisture content (%) was carried out according to AOAC International methods 950.46 (AOAC, 2006).

**2.2.2 Colour measurement**

The colour of ripened sausages was analysed according to the International Lighting Commission (CIE) system using the parameters *L\** (lightness), *a\** (redness), *b\** (yellowness) with a colorimeter CM-600d (Konica Minolta, Tokyo, Japan) equipped with the software SpectraMagic NX (Konica Minolta, Tokyo, Japan). The measurements were taken on different points on both surfaces of three 2-cm-thick slices of each sausage.

**2.2.3 Lipid oxidation**

The lipid oxidation level of ripened sausages was determined by thiobarbituric acid reactive substances (TBARs) as reported in Rosmini *et al.* (1996). The results were expressed in mg of malondialdehyde (MDA) per kg of ripened sausages, using a calibration curve obtained using 1,1,3,3-Tetraethoxypropane (TEP) as standard (Sigma-Aldrich, Germany) (0.01 M) at different concentrations (0.22 mg/kg - 2.2 mg/kg).

**2.2.4 Nitrosamines content**

Nitrosamines were extracted according to the method described by Cintya *et al.* (2019). The NAs level was determined using Ultra High Performance Liquid Chromatography (UHPLC) (Thermo Fischer Scientific, Waltham, USA) as reported in Al-kaseem *et al.* (2014). Wavelengths of 231 nm was used for absorbance detection and the quantification of the nitrosamines was obtained using the EPA 521 standard (Nitrosamine Mix, Supelco, Bellefonte, USA). The results were expressed in μg/kg of ripened sausage.

**2.2.5 Statistical analysis**

Data were subjected to Two-Way ANOVA followed by the Tukey’s HSD test considering the dose of OLE and the dose of nitrate and nitrite added as independent variables. Significant differences were determined at *p<*0.05 by Minitab Statistical Software (Minitab Inc., State College, USA).

**2.3 Shelf-life prediction of ripened sausages with OLE during storage in MAP condition at 4°C**

The extension of shelf-life of the meat-based products is another challenge of the meat industry and ripened sausages were increasingly marketed in vacuum or in Protected Atmospheres Packaging (MAP), as whole piece or sliced. MAP is one of the preservation and packaging solutions being employed to meet customer demand for food and its use for processed meat has greatly grown in recent years (Ameer *et al.,* 2022). In the third experimental step of this research a shelf-life study of ripened sausages with OLE added was performed. To this aim, samples F1, F4 and F5 (Table 1) carried out during *scale-up* were considered. In this way, the effect of OLE when it is added alone and in combination with nitrates and nitrites in comparison to a sample with only synthetic additives added should be highlighted during the storage. At the end of ripening period, sausages were sliced, placed in sterile plastic trays (95 × 10 mm), and packed in MAP condition using a gas mixture 70:30 N2/CO2 and a plastic film composed of orientated polyamide/polypropylene (OPA/PP). All packs were stored at 4 °C for 80 days and examined at 0 days (T0), 10 days (T10), 20 days (T20), 40 days (T40), 60 days (T60) and 80 days (T80). The evolution of the oxidative degradation carried out with TBARs-test was considered as target phenomena to predict shelf-life of ripened sausages with OLE.

# **3. Results and Discussion**

**3.1 Preliminary trial of ripened sausages production with OLE addition in laboratory scale**

***Microbiological analysis, nitrate and nitrite residual content***

The aims of the preliminary trials were to assess the hygiene and safety parameters and the nitrate residual content in the different formulation. At the end of ripening period in all sausages with OLE added, all the microbiological parameters evaluated were within the law limits Reg. (CE) No. 1441/2007 (data not shown). A possible role of OLE in exerting an antimicrobial effect can be assumed since also in the samples without synthetic additives no growth was found. This could be due to the direct inhibitory action of OLE and the presence of polyphenolic compounds with antimicrobial properties. Furthermore, an effectiveness reduction of residual nitrate and nitrite level was obtained. In fact, the highest residual values were found in the control sample with only nitrate and nitrite. On the contrary, in the samples with only OLE added residual nitrate and nitrite were lower than limit of detection or not detected (data not shown).

**3.2 Ripened sausages with OLE added produced during *scale-up***

The main aims of this second study were the standardisation of the production process of ripened sausages and to highlight the evaluation of OLE addition on the lipid oxidation and the presence of NAs at the end of ripening period, considering the production with industrial equipment.

***Weight loss, water activity (aw), pH, moisture content***

The results of weight loss (%), moisture (%), pH and aw of the ripened sausages are shown in Table 2. As regards moisture and activity water (aw), the presence of nitrate and nitrite in samples F2, F4 and F5 resulted in a significant reduction compared to samples F1 and F3 with only OLE and without any type of additives, respectively. This could be due to the osmotic dehydration induced by the presence of these synthetic additives (Deng *et al.,* 2021).

**Table 2** *Mean value, standard deviation, and results of the statistical analysis (two-way ANOVA) of weight loss, moisture, pH and water activity (aw) of the ripened sausages.*

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
|  | **F1** | | **F2** | **F3** | **F4** | **F5** | ***p-value*** |
| **Weight loss (%)** | | 41.61±0.42B | 48.56±0.44A | 42.03±1.04B | 48.08±1.18A | 47.85±1.00A | OLE=0.056  NO2-NO3=0.008 |
| **Moisture (%)** | | 29.85±0.65A | 24.60±0.27B | 30.51±1.00A | 22.46±0.38C | 23.88±0.89B | OLE=0.057  NO2-NO3<0.001 |
| **pH** | | 6.04±0.02B | 6.04±0.03B | 6.13±0.02A | 6.18±0.03A | 6.02±0.02B | OLE=0.003  NO2-NO3<0.001 |
| **aw** | | 0.85±0.01A | 0.83±0.01B | 0.86±0.00A | 0.83±0.00B | 0.82±0.01B | OLE=0.081  NO2-NO3<0.001 |
| Different letters in the same raw indicate significant differences at *p<*0.05. | | | | | | | |

This trend has been also confirmed by weight loss which is generally related to these two latter parameters. In fact, the presence of nitrate and nitrite, both alone (F4) and in combination with the OLE (F2, F5) resulted in significantly higher weight loss than the samples where they were not added. Considering pH, the presence of the OLE (F1, F2, F5) resulted in a significant decrease of this parameter. This could be due to the presence of fermentable carbohydrates of the OLE that could promote the metabolism of lactic acid bacteria which induce acidification (Flamminii *et al.,* 2019).

***Colour analysis***

The results of colour analysis are reported in Table 3. The absence of nitrate and nitrite in samples F1 and F3 induced a significant reduction of *a\** index, probably due to the lack of synthesis of the nitroso-myoglobin which determine the stability of the typical red colour of these products. On the contrary, sample F4 with only with nitrate and nitrite showed significantly higher value than all other samples.

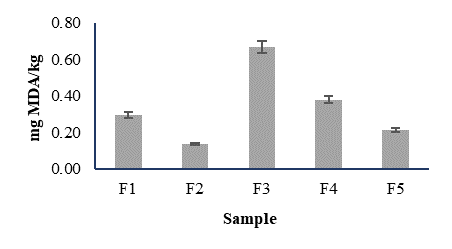
**Table 3** *Mean value, standard deviation, and results of the statistical analysis (two-way ANOVA) of lightness (L\*), redness (a\*) and yellowness (b\*) of the ripened sausages.*

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
|  | **F1** | | **F2** | **F3** | **F4** | **F5** | ***p-value*** |
| **Lightness (*L\**)** | | 36.61±0.24A | 34.18±0.08B | 36.37±0.07A | 33.59±0.29C | 34.68±0.42B | OLE=0.104  NO2-NO3<0.001 |
| **Redness (*a\**)** | | 6.43±0.69D | 8.07±0.18B | 5.74±0.16D | 8.78±1.39A | 6.87±1.05C | OLE=0.085  NO2-NO3<0.001 |
| **Yellowness (*b\**)** | | 6.85±0.42BC | 6.64±0.27C | 8.10±0.92A | 7.65±0.64B | 6.63±0.81C | OLE=0.144  NO2-NO3=0.033 |
| Different letters in the same raw indicate significant differences at *p<*0.05. | | | | | | | |

Considering yellowness (*b\**), generally associated with lipid oxidation, sample F3 without any type of additive added showed significantly higher value than all other samples. On the contrary, sample F2 and F5 with both types of additives at different concentration showed significantly lower values. Furthermore, no significant differences emerged compared to F1 with only OLE added. Differences emerged among different formulations could be due to the different lipid oxidation level of ripened sausages which could be limited by the presence of OLE both alone (F1) and in combination with nitrate and nitrite (F2, F5).

***TBARs-test***

The antioxidant effect of the OLE was also highlighted by TBARs-test results reported in Figure 1. Sample F3 was the most oxidised with an MDA mean content of 0.66 mg/kg sample. In contrast, sample F2 with OLE and nitrate/nitrite at the highest doses showed the lowest value. In addition, sample F1 with only OLE showed significantly lower MDA content than sample F4 with only nitrate and nitrite. These results therefore could confirm the effectiveness of OLE both alone and in combination with nitrate and nitrite in retarding the oxidation process of the ripened sausages thanks to the antioxidant effect of OLE, particularly richness in oleuropein which can act as scavenger against free radicals (Hassen *et al.,* 2015).



A

B

C

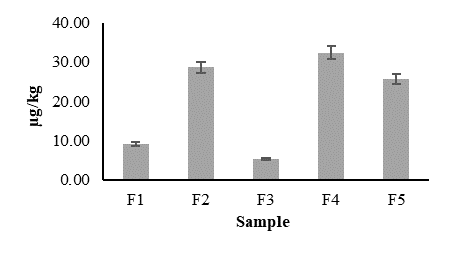
D

E

**Figure 1** *Results of the TBARs-test (mg MDA/kg) of the ripened sausages. Different letters indicate significant differences at p<0.05.*

***Nitrosamine content***

In Figure 2 the total nitrosamines content is reported. As it was expected the highest significant value was found in sample F4 with only nitrate and nitrite. On the contrary the lower values were found in samples F1 and F3 without synthetic additives added. The reduction of the OLE and nitrate/nitrite doses in sample F5 induced a significant reduction compared to sample F2 at the highest doses. These results could highlight the effectiveness of the extract in the reduction of nitrosamines formation in sausages at the end of the ripening period. In fact, it was demonstrate that polyphenols could reduce nitrite to hydroxyl groups in their structure to release hydrogen to react with free radical, blocking the chain reaction of free radicals, thus reducing the formation of nitrosamines (Gao *et al.,* 2022). At the same time, although at significant lower amount, the presence of these compounds in samples F1 and F3, could be due to the presence of the OLE which naturally present little amount of nitrite and contamination events that occurred during processing, respectively.



E

A

D

C

B

**Figure 2** *Results of the total nitrosamines content (µg/kg) of the ripened sausages. Different letters indicate significant differences at p<0.05.*

**3.3 Shelf-life prediction of ripened sausages with OLE during storage in MAP condition at 4°C**

Kinetic models are a useful tool for the control and prediction of quality indices changes in foods. They are used to describe the formation of undesired compounds, aggregation in texture formation and inactivation of enzymes and microorganisms (Zhang *et al.,* 2021). This approach could be helpful to define the shelf-life of foods and thus the “best before” date to be reported on the label (Conte *et al.,* 2020). In meat products in which no contaminant microbial grown was observed (as in our samples) the evolution of the oxidative degradation is considered as target phenomena that prejudice the consumer acceptability of the products and so their shelf-life.

The first step concerned the identification of the most appropriate indicators leading to quality loss followed by the definition of the relevant acceptability limit. As regards ripened sausages, TBARs is considered one of the main analytical indices used to monitor the evolution of oxidation during storage and the acceptability limit is equal to 1 mg MDA/kg (Ockerman, 1976) that was reached in F4 samples after 70 days of storage (data not showed). In the next step, the reaction order of quality index was estimated based on R2 obtained from MDA levels change of F1 (R2=0.994) and F5 (R2=0.976) as a function of the storage time (Figure 3). These results showed that the chose quality index fitted with the zero-order reaction model. Finally, data describing the evolution of the oxidative indicator as a function of time were submitted to modelling according to the equation (1) reported in Manzocco *et al.* (2016):

(1)

where SL is shelf life; Ilim is the oxidative value corresponding to the previously defined acceptability limit; I0 is the value of the oxidative indicator just after sausages production; k is the rate constant. Based on the kinetic models obtained, the acceptability limit defined for lipid oxidation of the F1 and F5 samples will be reached, respectively, after 99 and 157 days of storage in MAP at 4 ºC.

**4. Conclusions and Future Perspectives**

To conclude this oral communication, even if is just an extract of the whole studies, showed the effectiveness of using the OLE for the reduction of nitrate and nitrite level in the ripened sausages through three different experimental steps. Together with an effective reduction of the nitrate/nitrite residual content, the replacement of the synthetic additives with the extract did not affect the hygiene and safety parameters of the sausages produced at laboratory scale. Based on these results, a *scale-up* approach was also experimented. In this case, the results of colour parameters, TBARs-test and total nitrosamines content showed the antioxidant effect of the extract. In addition, to predict the time needed to exceed the limit of level of incipient rancidity ripened sausages during storage, a shelf-life study was carried out. In this case, the kinetic model obtained confirm the effectiveness of using OLE as an alternative natural additive to improve and prolong the shelf-life of ripened pork sausages.

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